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BRIEF COMMUNICATION

Phytosulfokine stimulates cell divisions in sugar beet (*Beta vulgaris* L.) mesophyll protoplast cultures

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Abstract The aim of this work was to improve plating efficiency of sugar beet mesophyll protoplast cultures. Preliminary experiments showed that cultures of good quality, viable protoplasts were obtained in rich media based on the Kao and Michayluk formulation and with the calcium alginate as an embedding matrix. Nevertheless, in these cultures cell divisions were either not observed or very seldom confirming earlier reported recalcitrance of sugar beet protoplasts. The recalcitrant status of these cultures was reversed upon application of exogenous phytosulfokine (PSK)—a peptidyl plant growth factor. The highest effectiveness of PSK was observed at 100 nM concentration. Plating efficiencies obtained in the presence of PSK reached approximately 20% of the total cultured cells. The stimulatory effect of phytosulfokine was observed for all tested breeding stocks of sugar beet. Our data indicate that PSK is a powerful agent able to overcome recalcitrance of plant protoplast cultures.

Keywords Sugar beet · Protoplast culture · Phytosulfokine · PSK · Cell division

Introduction

Despite the substantial progress made over the last two decades sugar beet is still considered to be a recalcitrant species with respect to protoplast culture (Majewska-Sawka and Münster 2003). Major improvements were introduced with the use of (1) *n*-propyl gallate (nPG)—a lipoxygenase inhibitor included both in the isolation and culture media (Krens et al. 1990), (2) protoplast embedding—especially in calcium alginate (Schlangstedt et al. 1992; Hall et al. 1993), (3) feeder systems exploiting nurse protoplasts or cells from suspension culture (Hall et al. 1993) and (4) stomatal guard cells or hypocotyl-derived friable callus as protoplast sources (Hall et al. 1997; Dovzhenko and Koop 2003). However, the latter two improvements involve rather complex manipulations and therefore, they are not well-suited for the routine laboratory practice. Moreover, in most reported cases responsiveness of the sugar beet protoplast culture was shown to be strictly genotype-dependent limiting its use for purposes where wider gene pool is required (Jeżdżewska et al. 1995). For these reasons the problem of sugar beet protoplast recalcitrance is still addressed in scientific efforts—both in terms of its biochemical basis and possible circumvention (Wiśniewska and Majewska-Sawka 2007, 2008). Chemical factors, which have been reported to increase plating efficiency of sugar beet protoplasts, include: (1) already mentioned *n*-propyl gallate, (2) exogenous polyamines and (3) arabinogalactan proteins. However, none of them is sufficient enough to overcome recalcitrance of sugar beet leaf protoplast culture.

In recent years peptide hormones have been recognized as important regulatory molecules in plant cells (Ryan et al. 2002, Matsubayashi 2003). One of them is phytosulphokine (PSK) isolated for the first time from asparagus mesophyll cell cultures by Matsubayashi and Sakagami (1996). PSK is a five amino acid peptide containing two posttranslationally sulfated tyrosine residues. This molecule results from cleavage of the precursor proteins. Tyrosine sulfation of the preprophytosulfokine is catalyzed

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by the tyrosylprotein sulfotransferase in the Golgi apparatus (Hanai et al. 2000). PSK is known to promote mitogenic activity—this effect was observed both in low-density cell suspensions and in callus cultures. It was also shown to stimulate somatic embryogenesis, tracheary element differentiation and formation of adventitious roots. Moreover, PSK is involved in density-dependent pollen germination, cellular longevity and exhibits protective effect against high night temperature (Lorbiecke et al. 2005 and ref. therein, Matsubayashi et al. 2006). Its effects were observed in plants as diverse as dicotyledons, monocotyledons and conifers.

Due to its universal character PSK is a good candidate supplement for plant in vitro cultures—similarly to widely exploited auxins, cytokinins and gibberellins. The only protoplast cultures, which have been shown to respond to PSK with enhanced proliferation activity, were derived from cell suspensions of rice (Matsubayashi et al. 1997). In this paper we report a very high positive impact of PSK on otherwise extremely recalcitrant sugar beet leaf protoplast culture.

Materials and methods

Donor plants

Six different breeding stocks of sugar beet (*Beta vulgaris* L.) were used as donors for protoplast isolation—male-sterile lines: S 00 1073-1, S 02 131, S 02 203, S-79-9-2 as well as male-fertile lines: 21-46-4-5 and 24-05-1-3. These materials had doubled haploid origin and were produced through in vitro culture of unpollinated ovules in sugar beet breeding company KHBC Straszków, Poland. Protoplasts were isolated from established shoot cultures which were maintained by transferring individual shoots in 2 weeks intervals on fresh BCM medium—MS including vitamins (Murashige and Skoog 1962) supplemented with 0.3 mg l^{-1} N^6-benzylaminopurine (BAP), 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA), 0.3 mg l^{-1} thiamin-HCl, 3% (w/v) sucrose and 0.7% (w/v) agar (Plant Propagation Lab Agar, Biocorp), pH = 5.8. To prevent endogenous bacterial contaminations often found in sugar beet tissue cultures cefotaxime was filter-sterilized (0.22 μm, Millipore) to the autoclaved medium to the final concentration of 200 mg l^{-1}. Shoot cultures were grown in a climate room at 26 ± 2°C, under light intensity of 55 μmol m^{-2} s^{-1} with 16-h photoperiod.

Protoplast isolation and purification

Protoplasts were isolated from leaves of the 2 week-old shoot cultures (Fig. 1a). 5–10 leaves (approx. 1 g) were cut into fine pieces in a 9 cm glass Petri dish containing 8 ml of the plasmolysis solution (0.5 M mannitol) and incubated for 1 h at 26°C in the dark. Then the tissue was macerated in the enzyme mixture containing of 1% (w/v) cellulase Onozuka R-10 (Duchefa), 1.5% (w/v) macerozyme R-10 (Serva), 30 mM 2-(N-morpholino) ethanesulfonic acid (MES, Sigma) and 0.45 M D-sorbitol (Sigma) in cell protoplast wash (CPW) solution (Frearson et al. 1973). Maceration was performed overnight at 26°C with continuous gentle shaking (30 rpm). Quality of the released protoplasts was examined under inverted microscope (Axiovert S100, Zeiss). Subsequently, the suspension was passed through a 80 μm nylon sieve (Millipore) in order to remove undigested tissue and then centrifuged at 1,000 rpm for 5 min. The pellet was resuspended in 8 ml of 0.5 M sucrose, 1 mM MES and overlaid with 2 ml of W5 solution (Menczel et al. 1981). After centrifugation at 1,200 rpm for 10 min pure and viable protoplasts were concentrated on the interphase between the two solutions. Collected with a Pasteur pipette protoplasts were then washed twice: first in 10 ml of the W5 solution and then in 10 ml of the culture medium (see section Protoplast culture). After each wash the protoplasts were spun down at 1,000 rpm for 5 min.

Protoplast embedding

After purification the protoplasts were suspended in approx. 1 ml of the culture medium and their yield was determined using a Fuchs-Rosenthal hemocytometer. After that cell density was brought to 8×10^5 per ml. Two systems of protoplast immobilization were tested: embedding in thin layers of calcium alginate (Ca-alginate) and embedding in agarose beads. In the former method a modified protocol of Damm et al. (1989) was used. First, equal volumes of the protoplast suspension and an autoclaved solution of 2.8% alginic acid sodium salt (Sigma), 0.4 M mannitol were mixed carefully. Subsequently aliquots (approx. 300 μl) of the protoplasts/alginate mixture were spread as thin layers in 6 cm Petri dishes on 1% (w/v) agar (Biocorp) containing 20 mM $CaCl_2$ and 0.4 M mannitol. After 1 h incubation at room temperature solidified alginate-protoplast layers were transferred into 6 cm Petri dishes containing 4 ml of the culture medium. In the second immobilization system two types of low-gelling temperature agarose were used. These were filter-sterilized solutions of 0.6% (w/v) agarose type VII (Sigma) and 1.2% SeaPlaque agarose (Duchefa) in the culture medium. Equal volumes of the protoplast suspension and agarose solution (of approx. 40°C) were mixed and seven 50 μl-aliquots of the resulting mixture were dropped onto the bottom of a 6 cm Petri dish. After agarose solidification 4 ml of the culture medium was added. In all immobilization methods the final density of the embedded protoplasts was 4×10^5 per ml.

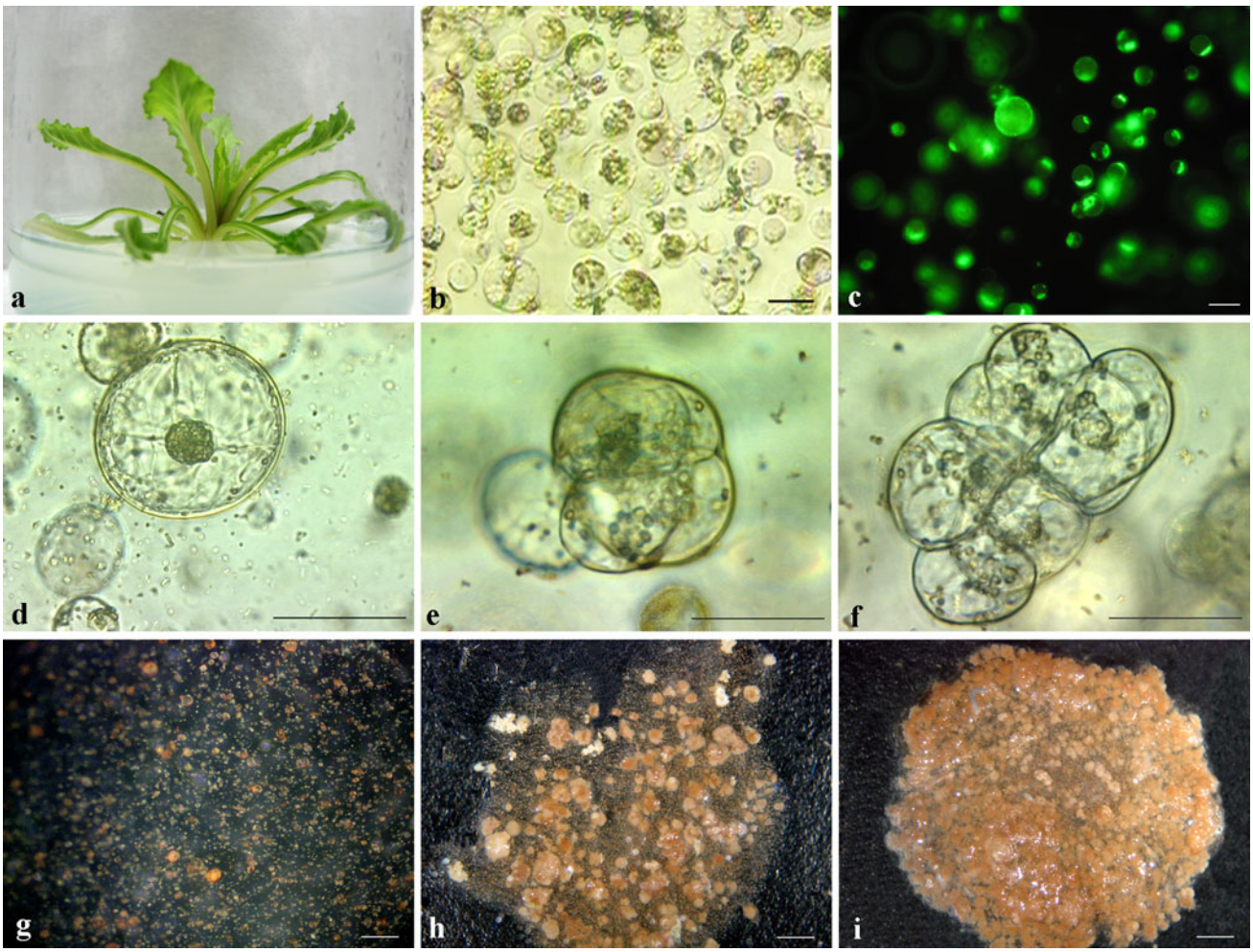

Fig. 1 Different stages of sugar beet leaf protoplast isolation and culture. **a** Donor plant from 2 week-old shoot culture. **b** Freshly isolated leaf protoplasts. **c** Leaf protoplasts stained with fluorescein diacetate (FDA). **d**, **e**, **f** 7-day cultures grown in calcium alginate and medium 2B with 0, 10 and 100 nM phytosulfokine (PSK), respectively. **g**, **h**, **i** Protoplast-derived macrocalli in 2-month cultures grown in calcium alginate and medium 2B with 0, 10 and 100 nM PSK, respectively. *Scale bars* represent either 50 μm (**b**–**f**) or 0.5 cm (**g**–**i**)

Protoplast culture

In preliminary experiments the embedded protoplasts were cultured in the three different liquid culture media. Medium 1B contained: macro-, microelements and vitamins according to MS (Murashige and Skoog 1962), 12% (w/v) sucrose, 0.5 mg l^{-1} BAP and 1 mg l^{-1} NAA. Medium 2B contained: macro-, microelements and organic acids according to KM (Kao and Michayluk 1975), vitamins as in B5 (Gamborg et al. 1968), 7.4% glucose, 250 mg l^{-1} casein enzymatic hydrolysate (Sigma), 8 mg l^{-1} putrescine (Sigma), 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg l^{-1} zeatin. Medium 3B was composed as 2B with addition of KM vitamins, 2% (v/v) coconut water and 15% (w/v) sucrose. In further experiments media 2B and 3B were supplemented with either 10, 100 or 1,000 nM phytosulfokine-α (PSK). PSK was synthesized by Peptide Institute, Inc. (Osaka, Japan). All media contained cefotaxime at concentration of 200 mg l^{-1}.

Protoplast cultures were incubated at 26°C in the dark until callus macrocolonies were formed (approx. 2 months). After each 10 days of culture the medium was replaced with its fresh portion in order to remove toxin substances produced by the cells.

All solutions and media used for protoplasts isolation, washing and culture contained 10 μM *n*-propyl gallate (nPG, Sigma) as an antioxidant. The culture media (1B, 2B, 3B) and enzyme solution were adjusted to pH 5.6 and filter-sterilized (0.22 μm, Millipore). All other solutions and culture media were adjusted to pH 5.8 and autoclaved (21 min, 121°C, 0.1 MPa).

Microscopic observations and data analysis

Protoplast viability was assessed by staining with fluorescein diacetate (FDA) according to the protocol of Anthony et al. (1999). For this purpose 60 µl of 0.3% FDA acetone stock was added to 4 ml of the culture medium to yield the FDA working solution. 100 µl of this solution was added to a Petri dish with the protoplast culture. Apple-green fluorescence of viable cells was examined under Zeiss Axiovert S100 microscope equipped with the appropriate filter set ($\lambda_{Ex} = 485$, $\lambda_{Em} = 515$ nm) (Fig. 1c). Plating efficiency was expressed as the number of divided cells per total number of plated protoplasts multiplied by 100.

Data were collected in three independent experiments. Each treatment was set up in three replicates. For a single Petri dish both viability and plating efficiency counts were carried out in five microscopic fields with 100–400 cells. The mean values and standard errors were calculated. The overall effect of treatments was assessed using analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test. Significant differences were expressed at $P \leq 0.05$. The computations were performed using Statistica software ver. 8 (StatSoft. Inc.).

Results

To improve plating efficiency of sugar beet protoplast culture, the following parameters were assessed with respect to their impact on protoplast viability and colony formation: (1) type of protoplast embedding agent, (2) type of culture medium and (3) concentration of phytosulfokine (PSK) in the culture medium.

Protoplasts were efficiently released from the leaf tissue after 14–16 h of incubation in the enzyme solution. Protoplast yields ranged from $2.3 \pm 0.5 \times 10^6$ per g fresh weight (FW) for line S 02 131 to $2.8 \pm 0.9 \times 10^6$ for line S 02 203. Freshly isolated protoplasts were spherical in shape, varied in size and contained numerous differentially oriented chloroplasts (Fig. 1b).

Effect of embedding matrices and culture media on protoplast development

In these experiments protoplasts were isolated from three male-sterile lines: S 00 1073-1, S 02 131 and S 02 203. These breeding stocks as well as different immobilization agents and culture media were examined with respect to their effect on protoplast viability and division potential.

Protoplast viability in the first hour of culture was relatively low and varied from 38% for line S 00 1073-1 to 49% for line S 02 203 (Table 1). On the next day of culture only a slight decrease in cell viability was observed while on the second day its value was approximately 10% lower than the one recorded immediately after isolation. In general, no significant differences were observed in viability of protoplasts isolated from different breeding stocks. Similarly, immobilization of protoplasts in Ca-alginate, agarose type VII as well as in SeaPlaque agarose resulted in comparable values of protoplast viability (Table 1). Despite the fact that in the first, 24th, and 48th hour of culture the highest viability was observed for the cells embedded in Ca-alginate it appeared to be not statistically significant. Conversely, the protoplast viability was clearly dependent on the applied culture medium (Fig. 2). More viable cells were observed in rich mineral-organic media 2B and 3B which were based on the Kao and Mychayluk formula. Medium 1B based on the Murashige and Skoog recipe resulted in approximately half-reduced cell viability. After 5 days of culture only protoplasts embedded in Ca-alginate became larger and changed their shape from circular to elliptical indicating reconstruction of the cell wall. At that time the cells exhibited densely organized

Table 1 Viability (%) of protoplasts isolated from three male-sterile breeding stocks and immobilized in three different embedding matrices

	Age of culture (h)		
	1	24	48
Donor stock			
S 00 1073-1	38.3 ± 6.0	37.8 ± 5.0	29.1 ± 3.8
S 02 131	45.7 ± 4.7	42.0 ± 3.8	34.2 ± 4.8
S 02 203	48.7 ± 4.3	46.7 ± 3.3	38.0 ± 4.2
Embedding matrix			
Ca-alginate	46.7 ± 6.3	44.1 ± 5.5	38.2 ± 5.5
Agarose type VII	45.7 ± 4.6	42.0 ± 3.9	29.1 ± 3.5
SeaPlaque agarose	40.3 ± 4.6	40.3 ± 2.9	33.9 ± 3.4

Data represent means ± standard errors from three independent experiments (each with three replicates)

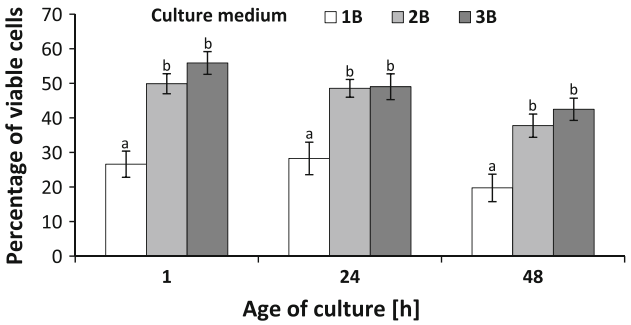

Fig. 2 Viability of protoplasts cultured in three different media. Bars represent means ± SE obtained for three breeding stocks and three embedding matrices. Means denoted with *different letters* are significantly different ($P \leq 0.05$)

cytoplasm and most of their chloroplasts were no longer visible. First cell divisions were observed sporadically after approximately 7 days of culture—but only for protoplasts isolated from line S 02 203, embedded in Ca-alginate and cultured on medium 2B. Further development of these cultures was arrested after next two or three cell divisions.

Response of protoplast cultures to phytosulfokine (PSK)

In these experiments protoplasts isolated from line S 02 203 were embedded in Ca-alginate layers and cultured in media 2B and 3B supplemented with either 10, 100 or 1,000 nM phytosulfokine. In parallel, controls without added PSK were also cultured. Neither the PSK concentration nor the type of culture medium influenced protoplast viability—this parameter was also relatively stable in subsequent points of evaluation (Table 2). After 7 days of culture first cell divisions were observed only in the presence of PSK (Fig. 3). When PSK was applied in 10 nM concentration only 1% of dividing cells was observed while in the presence of 100 nM PSK plating efficiency was about fivefold higher. The stronger effect of 100 nM PSK was also confirmed in the 14th and 21st day of culture—the observed values of plating efficiency reached 13 and 19%, respectively. Upon increase to 1,000 nM concentration further stimulation of cell divisions was not observed—this concentration of PSK was approximately as effective as 10 nM. As a result of the phytosulfokine application the protoplast-derived microcolonies were formed (Fig. 1d–f). After 6 weeks of culture in the Ca-alginate layers callus macrocolonies were clearly visible with the naked eye. At that time it also became apparent that the higher PSK concentration resulted in a faster growth of microcalli (Fig. 1g–i). There were no significant differences in plating efficiency between the two applied culture media (data not shown).

Table 2 Viability (%) of protoplasts isolated from line S 02 203, embedded in Ca-alginate and cultured in two media supplemented with different concentrations of phytosulfokine (PSK)

Culture medium	PSK concentration (nM)	Age of culture (h)		
		1	24	48
2B	0	64.4 ± 4.7	62.0 ± 1.7	58.2 ± 1.3
	10	65.8 ± 2.4	61.0 ± 1.9	58.0 ± 0.6
	100	68.3 ± 1.3	64.0 ± 2.7	62.4 ± 2.4
3B	0	66.6 ± 4.0	60.0 ± 7.1	60.7 ± 3.3
	10	64.1 ± 5.4	58.1 ± 3.2	60.8 ± 4.6
	100	65.5 ± 4.4	64.5 ± 3.4	61.8 ± 4.4

Data represent means ± standard errors from three independent experiments (each with three replicates)

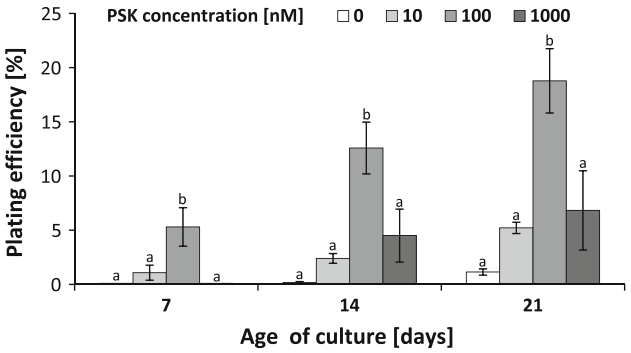

Fig. 3 Plating efficiency of protoplast cultures with different concentrations of phytosulfokine (PSK). The protoplasts were isolated from S 02 203 plants and embedded in Ca-alginate. Bars represent means ± SE obtained for two culture media (2B and 3B). Means denoted with *different letters* are significantly different ($P \leq 0.05$)

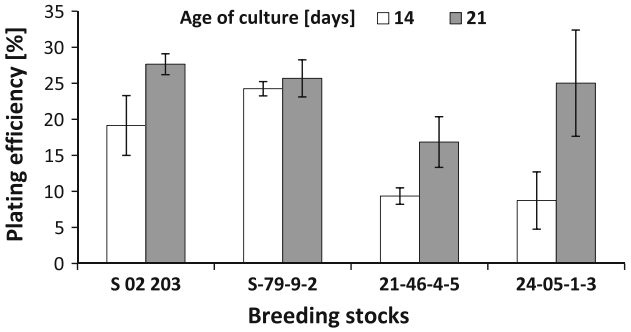

Fig. 4 Plating efficiency of protoplast cultures obtained for different sugar beet breeding stocks. The protoplasts were embedded in Ca-alginate and cultured in medium 2B supplemented with 100 nM phytosulfokine (PSK). *Bars* represent means ± SE

Genotype dependence of PSK action

For the purpose of this investigation mesophyll protoplasts were isolated from the two male-sterile (S 02 203, S-79-9-2) and two male-fertile (21-46-4-5, 24-05-1-3) lines, immobilized in the Ca-alginate layers and cultured in 2B medium supplemented with 100 nM PSK. After 2 weeks of culture plating efficiency varied from 9 (line 24-05-1-3) to 24% (line S 79-9-2, Fig. 4). 1 week later number of microcolonies increased ($P < 0.05$) and ranged from 17% for line 21-46-4-5 to 28% for line S 02 203. 2 weeks after protoplast isolation more cell colonies were observed in cultures derived from the male-sterile breeding stocks than in cultures obtained from the fertile lines ($P < 0.05$). After 3 weeks of culture the observed plating efficiency was similar for all studied breeding stocks. These data may indicate that protoplasts isolated from the male-sterile material responded faster to phytosulfokine application—however, it cannot be excluded that this variation is caused by other genotypic differences.

Discussion

Plant protoplasts have a range of biotechonological applications including somatic hybridization, somatic cybridization and gene transfer by means of either chemical treatment, microinjection or electroporation. They have also been used for organellar transplantation—in most cases uptake of chloroplasts and nuclei was reported (reviewed in Razdan 2003). The necessary prerequisite for these applications is an ability of plant protoplasts to reconstitute their cell wall and undergo mitotic divisions which results in callus formation and eventually shoot regeneration (Davey et al. 2005a). The problem is that for many species this developmental program is hard to achieve. Such recalcitrance of plant in vitro cultures is a widely known phenomenon—its importance was recognized from the very beginning of this research field (Benson 2000). With respect to protoplast cultures recalcitrance was noted for most monocotyledons, perennial woody species and legumes (Papadakis and Roubelakis-Angelakis 2002; Davey et al. 2010). This list can be extended to include sugar beet as well, at least with respect to protoplasts of mesophyll origin (Hall et al. 1993, Majewska-Sawka and Münster 2003). Obviously, such limitation affects utilization of plant protoplasts for the above-mentioned very potent downstream applications. As a consequence successful use of plant protoplast cultures has been limited to well-responsive species, mainly from families *Solanaceae* and *Brassicaceae* (Davey et al. 2005a). Accordingly, so far most reports on sugar beet protoplasts have been dedicated to optimization of culture conditions and hence reports on successful (ended up with regeneration of the modified plants) biotechnological utilization of these cultures are hardly existing.

An early improvement on the way to overcome protoplast recalcitrance was to exploit actively dividing cultured cells for protoplast isolation. Although very powerful this invention has an apparent limitation—it requires prior establishment of a suspension culture, a process which takes months and itself for many plant species is far from a routine (Mustafa et al. 2011). This approach was successfully used also in case of sugar beet allowing to reach plating efficiency of about 40% (Majewska-Sawka et al. 1994, 1997). For this species two alternative protoplast sources were devised. Hall et al. (1996, 1997) reported that sugar beet stomatal guard cells exhibited totipotency and provided protoplasts for which the mean plating efficiency of the best responsive accession reached almost 22%. Similar plating efficiencies were obtained by Dovzhenko and Koop (2003) with protoplasts isolated from friable regenerable callus. Both approaches proved to be very efficient—however, the former was not reproduced by other groups (Dovzhenko and Koop 2003) and the latter needs an additional investment in friable callus production which according to our experience is very difficult for some genotypes (data not shown). Here presented experiments deal exclusively with leaf protoplasts which in case of sugar beet are regarded as extremely recalcitrant. For this material reported plating efficiency ranged from 0.04 to 1.2% (Majewska-Sawka and Münster 2003). On the other hand mesophyll exhibits some important advantages as a donor tissue—it is easily available, does not require prior callus or suspension culture and its dedicated isolation protocols are relatively straightforward. Therefore, the use of leaf material would be especially desirable for applications in which large quantities of high quality protoplasts are required e.g. for fusion purposes.

Another very common way to overcome protoplast recalcitrance is to use cell culture supplements e.g. nonionic surfactants or artificial oxygen carriers which enhance physiological status of the cultured cells. Such factors are able to increase plating efficiencies even a few-fold (Davey et al. 2005b). In this report we show beneficiary effect of phytosulfokine (PSK) which represents a new class of supplements—the peptide growth factors. It is demonstrated that application of exogenous PSK is able to reverse the recalcitrant behavior of in vitro cultured cells. For the investigated here sugar beet mesophyll protoplasts the effect of PSK application was much more pronounced than caused by any other earlier studied culture supplement. We observed about 20-fold increase of plating efficiency over control lacking PSK which brings this parameter to the level observed by the authors who used the above-referenced alternative donor materials—suspension cultures, epidermis and callus. Phytosulfokine was not only launching proliferation activity—it was also essential for its maintenance. In no PSK controls even if cell divisions occurred they were soon arrested and as a result callus formation was not observed. Out of the three analyzed PSK concentrations, 100 nM exhibited the highest activity which was within the range observed in other in vitro systems (Matsubayashi and Sakagami 1996; Hanai et al. 2000) and indicated that in sugar beet protoplast culture one of the universal pathways of PSK action was initiated. All these data also suggest that recalcitrance may be associated with insufficient production of phytosulphokine in in vitro conditions. Obviously, this hypothesis will need biochemical verification, it will also be interesting to trace expression of PSK precursor protein(s) through subsequent stages of the protoplast culture.

Except PSK other parameters of our sugar beet protoplast cultures resembled those brought by the previous reports (see above). We compared three different embedding matrices and showed superiority of the thin alginate layers. This effect may be due to the fact that agarose (1) forming thicker layers than alginate hindered diffusion

between cells and medium or (2) contained impurities affecting proper protoplast development. It is also possible that sugar beet mesophyll protoplasts are to some extent heat-sensitive. Our experience confirms beneficiary effect of *n*-propyl gallate (nPG) although contrary to other reports we used it in concentration of 10 instead of 100 μM.

It seems that reasons for recalcitrance of protoplast cultures may be at least twofold. Firstly, they may be related to the stress exerted upon plant cells during protoplast isolation and subsequent culture. For example Papadakis and Roubelakis-Angelakis (2002) point at active oxygen species and oxidative stress as factors affecting regeneration potential of protoplasts. Secondly, recalcitrance may result from lack of developmental signals which are present when plants are in toto. The above categorization gains its support from nature of factors which help to overcome protoplast recalcitrance. For example, *n*-propyl gallate (nPG) seems to target the first category of phenomena through its antioxidant activity (Saleem and Cutler 1987; Krens et al. 1994). In turn, the stimulating effect of nurse cells results from the chemical signals they emit to cultured protoplasts (Hall et al. 1993, Davey et al. 2005b). It is obvious that phytosulfokine also falls into the second category of factors and once its action was even referred to as chemical nursing (Matsubayashi et al. 2004). As such PSK exhibits a few important advantages over classical nursing—(1) it is easily applicated—when using it the parallel culture of nurse cells is not required, (2) it is a defined compound, (3) its level can be precisely controlled and (4) it is a universal signal molecule suggesting that its utilization may help to overcome protoplast recalcitrance in other crops of vital economic importance like e.g. cereals. Here presented data show that in case of sugar beet mesophyll protoplasts phytosulfokine is able to boost plating efficiency of their culture to the level sufficient for downstream applications. The effect of PSK was observed for all studied sugar beet accessions although protoplast cultures obtained from the male-sterile lines responded faster to its application. The male-sterile and male-fertile accessions differ with respect to the carried cytoplasm—the former are S- and the latter N-cytoplasmic. Cytoplasmic influence on plant material in vitro performance would not be surprising as it was earlier noted for wheat, barley, maize, potato, *Medicago* and *Brassica* (Henry et al. 1994).

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